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Original communication

Determination of clozapine in hair and nail: The role of keratinous biological materials in the identification of a bloated cadaver case

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ABSTRACT

Keratinous biological materials, such as hair and nails, offer a substantially longer retrospective window of detection compared to other body fluids. Little research on drug analysis in nails is currently being conducted. In this study, the hair and nails from a bloated cadaver was analyzed. The study showed that the forensic toxicology results of keratinous biological materials could provide valuable clues for solving cases.

In this study, a method was developed for the extraction and analysis of clozapine from hair and nails. The keratinous bio-samples were washed and then pulverized using a freeze mill. After ultrasonic bath extraction, the supernatants were analyzed by ultra-performance liquid chromatography tandem mass spectrometer (UPLC–MS/MS).

The method presented in this study proved to be reliable, specific, selective and sensitive with high precision and accuracy. Clozapine was found in both hair and nails from a long term user's remains, even after serious decomposition. The mean concentration of clozapine in the hair was 322.9 pg/mg and 138.3 pg/mg in the nails. Toxicological results helped police narrow the scope of the investigation and improved the efficiency of the breaking of the case.

The findings of the present study demonstrated that the method can be used in forensic investigation. Toxicological results increased the efficiency of cadaver identification and the solving of the case. The study demonstrated that hair and nail analysis could provide vital clues for solving cases and showed the value of keratinous biological materials in the forensics field.

1. Introduction

Hair and nails are keratinous biological materials that have a similar likelihood of accumulating drugs. The major advantage of keratinous biological materials in drug analysis compared to body fluids is that they have a larger surveillance window. This makes keratinous biological materials an important detection tool for toxicologists.[1] There are many applications in the literature in which hair analysis was used to document historic drug use or exposure, such as suspicious death, discrimination between single and chronic exposure, and crimes committed under the influence of drugs.[2] Hair testing for drugs has been successfully performed several months after death even following exhumation.[3] Balabanova et al. used hair to identify drugs in Egyptian mummies.[4] Nicotine was recently reported to have been detected in hair samples of pre-Columbian mummies.[5] Similar to hair, nails provide a stable material for detecting drug exposure[6] and can be a complement to hair in the detection of drugs.

Clozapine is an atypical antipsychotic medication with high toxicity. It is a prescribed drug in China, and few people can come into contact with this drug. Cirimele et al. detected clozapine in hair using gas chromatography coupled to mass spectrometry,[7] Weinmann et al. used liquid chromatography-tandem mass spectrometry to analyze hair samples of psychiatric patients,[8] and Thieme et al. studied hair segments.[9] Some reports shown that clozapine could be detected in hair even it was collected from a grave.[10] The determination of clozapine in hair and nail from schizophrenic patients was researched in our previous study.[11,12]

This paper presents a simple and sensitive UPLC–MS/MS method to detect clozapine in hair and nails. The method was validated, and the hair and nail samples from a bloated, immersed cadaver in a case were analyzed.

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2. Case

A bloated cadaver was found at the floodgate of a reservoir in a town that is located in the estuary of the Yangtse River, China, on 27 January 2013. The investigated remains were from a male of unknown name. His wrists, ankles and neck were tied, and no other obviously lethal wounds were present. Clozapine was detected in the blood at 470 ng/ml, which was within the therapeutic concentration range (the therapeutic concentration in blood of clozapine is 102–771 ng/ml[13]).

Identification of this anonymous deceased was difficult. The town is located in the estuary, where the local hydrographic net is complicated (drainage density = 3.25 km/km^2),[14] and the remains floated in the river for a long time. This delay period made it even harder for police to identify the location where the body was placed in the water. The population of this area is very high (the average population density in 2011 was 978 people/km^2).[15] DNA testing and fingerprint matching were unable to confirm the deceased in this case. To narrow the search and to determine whether the deceased was a psychiatric patient with a long history of clozapine use, hair and nail samples were collected for extensive testing.

3. Methods

3.1. Chemicals and reagent

Clozapine and clozapine-d$_4$, 1 mg/ml and 100 μg/ml ampoules, respectively, were purchased from Cerilliant (Round Rock, TX). High performance liquid chromatography (HPLC) grade acetonitrile was purchased from Sigma–Aldrich (St.Louis, MO, USA). Ammonium acetate (HPLC grade) and formic acid (HPLC grade, 50%) were purchased from Fluka Chemical Co. (Buchs, Switzerland). The deionized water used in experiments was produced by a Millipore milli-Q water purification system (Millipore, MA, USA). All other referred organic reagents were of analytical-reagent grade.

3.2. UPLC–MS/MS conditions

An Acquity™ Ultra Performance LC system (Waters Corporation, Milford, MA, USA) equipped with a quaternary pump, vacuum degasser, auto sampler, and column over compartment was used. Chromatographic separations were performed on a Restek Ultra IBD column (50 mm × 2.1 mm, i.d., 5 μm, Bellefonte, PA). The column temperature was set to 25 °C and the total flow rate was set to 200 μl/min. The mobile phase consisted of 70% acetonitrile and 30% ammonium acetate buffer (20 mM ammonium acetate buffer with 0.1% formic acid, pH 4.0), and run time was 7.0 min.

A 4000 QTRAP (Applied Biosystems/MDS Sciex, Concord, ON, Canada) hybrid triple quadruple-linear ion trap mass spectrometer equipped with electro spray ionization (ESI) source was used to analyze the samples. Samples were analyzed in the Multiple-reaction monitoring (MRM), positive mode. The clozapine (m/z 327.3 > 296.2, 327.3 > 270.1) and clozapine-d4 (m/z 331.2 > 299.2) ion transitions were monitored. The ESI source temperature was 500 °C. The curtain gas flow was 30 psi; collision gas was 7 psi; ion spray voltage was 5000 V; and entrance potential was 10 V.

The Analyst 1.5 software (Applied Biosystems, USA) was used for instrument control and data acquisition.

3.3. Sample collection and preparation

Hair strands (6 cm long) were cut as close as possible to the scalp. The ends corresponding to the cut were marked. The original color of the hair strands was black, but they looked blanched from the long soaking time. Extraordinarily, the nail samples were collected from the free edge of each finger or toe. Unfortunately, the fingernail on the middle finger of left hand, the toe nail on the middle toe of the right foot, the little toe of the right foot, and the little toe of the left foot were lost from the rotting. All samples were separated and stored in clean paper packs at room temperature.

The blank hairs and nails were obtained from healthy volunteers with no drug history from our laboratory and used for selectivity studies and to create spiked samples for the validation of the analytical procedure.

The method of sample preparation was optimized in previous studies.[11]

Hair strands were divided into 6 segments of 1 cm long from root to end. The hair segments or nail clippings from each finger or toe (15 mg) were washed independently with 2 ml deionized water twice by vigorous shaking for 3 min; then the water residue from the two washes was combined and moved to a separate tube for later analysis. Next, 2 ml of ethyl acetate were added to the hair or nail samples in the tube and shaken vigorously for 3 min and repeated once. After that, all organic residues were combined and saved in another tube for later analysis.

After drying at room temperature, the hair or nail samples were pulverized using a freeze mill (6770 Freezer/Mill, SPEX CertiPrep, Metuchen, NJ). A stainless steel impactor with an agitation rate at 5cps for 3 min at liquid nitrogen temperature was used, followed by a 2 min cool-down time, and then grounding again for 3 min. Hair or nail powder (5 mg) was then sonicated in an ultrasonic bath (KQ-100B, Kunshan, PRC) for an hour with 900 μl mobile phase and 100 μl 5 ng/ml clozapine-d$_4$. The ultrasound frequency was 40 kHz, and the power output of ultrasonic bath was 100 W. The water in the ultrasonic bath was changed periodically to avoid a temperature increase. The mixture was then centrifuged at 12 000 rpm for 5 min; the supernatant was injected into the UPLC–MS/MS system for analysis.

The preparation methods for the aqueous and organic wash residues were referred to as Tsanaclis L[16] and Hill V,[17] respectively. The aqueous or organic wash residues dried unaided and were reconstituted in 1000 μl of the mobile phase when the drying was complete. The reconstituted wash residues were analyzed as biological samples using UPLC–MS/MS. The analyte concentrations in the wash residue (W) and in the keratinous sample (K) were determined, and the results used to calculate the W/K ratio for each analyte in the samples.

3.4. Validation

Validations of the method were performed on blank hair and nail samples. According to EURACHEM guidelines,[18] the selectivity, linearity, accuracy and precision of the method was validated. Quality control (QC) samples (50, 200, and 750 pg/mg for clozapine) were included in each analytical batch to check calibration, accuracy and precision. All QC and calibration samples were prepared by spiking drug-free hair and nail samples with methanol working standard solutions and stored at −4 °C.

The selectivity of the method was confirmed by analyzing 6 different samples of blank hair and 6 different samples of blank nail. Detection and quantitation limits meet the requirements for practical examination.

Calibration curves were prepared and analyzed on four consecutive days. Linearity was determined from the calculation of a linear regression fit from the peak area vs. concentration plot for seven standard solutions spiked in blank matrices (10, 50, 100, 200, 500, 750 and 1000 pg/mg) using linear least squares methodology and analysis of the respective response factors.

The accuracy and precision were based on three different concentrations of QC samples. The accuracy was calculated as the

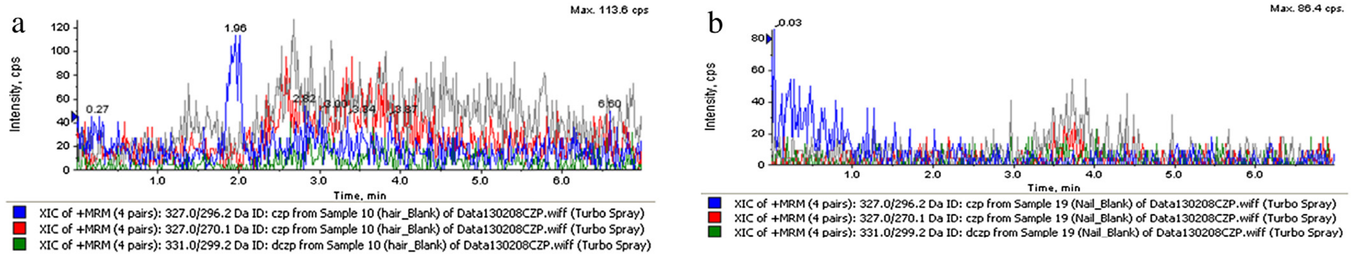

Fig. 1. MRM chromatograms of blank human hair (a) and blank human nail (b).

percentage of the measured concentration from the nominal concentration. The precision was calculated as the percentage coefficient of variation (CV%) within the assays (intra-day) and between assays performed on different days (inter-day).

As described by Matuszewski et al.,[19] the assessment of the matrix effects is based on a comparison of the peak areas obtained from the analysis of two sets of samples: set A consists of the standard solutions, and set B consists of the blank matrices with analyte spikes at the same concentration levels. The matrix effect $ME\% = B/A \times 100\%$ was investigated using a QC solution. Each level was prepared in 6 different matrices.

4. Result and discussion

4.1. Method and validation

The specificity of the method was evaluated using the blank hair and nail samples. There were no detectable interferences observed at the mass transitions and retention times of the clozapine and clozapine-d_4. Fig. 1 shows the representative mass chromatograms of the blank human keratinous matrix. Fig. 2 shows chromatograms of hair and nail of the deceased.

The intra-run accuracy and precision were determined from 6 replicate measurements of a QC sample at each concentration within a day. The inter-run accuracy and precision were determined over four days. For the hair samples, the precision at all concentration were less than 5.8%, while it was less than 7.0% for the nail samples. More detailed information was given in Table 1.

The matrix effect on the determination of clozapine in keratinous biological materials was lower than 20%, as shown in Table 1. The matrix effect of the hair was similar to that of the nail. In conventional preparations of keratinous biological materials,[6] the sample was extracted and concentrated. In our preparation process, the sample was diluted 200 times. The dilution makes the matrix effect maintained at a lower level. Similar preparation methods have successfully been applied to detect benzodiazepines drugs[20] and opiate abuse[21] in hair in our laboratory.

The calibration curves for human hair and nail were constructed using 7 non-zero hair or nail calibrators, as previously mentioned. The calibration equation was derived from four individual calibration curves from four validation batches with $1/x^2$ weighting. Table 2 shows the average calibration equation, determination coefficients, and the calibration curve range of the hair and nail samples. The limit of quantitation (LOQ) is the lowest concentration of the linear range, which is 10 pg/mg for hair or nail.

The method was characterized as requiring less sample and having a simple operation with rapid results and high sensitivity. The usual amount of keratinous materials was 10–50 mg,[6,22] but in some cases, the sample is difficult to obtain and very infrequently, the sample amount used in the method is 5 mg (almost equivalent to the weight of the nail clippings obtained from a single finger). The sensitivity of the method can meet the detection requirements of most cases.

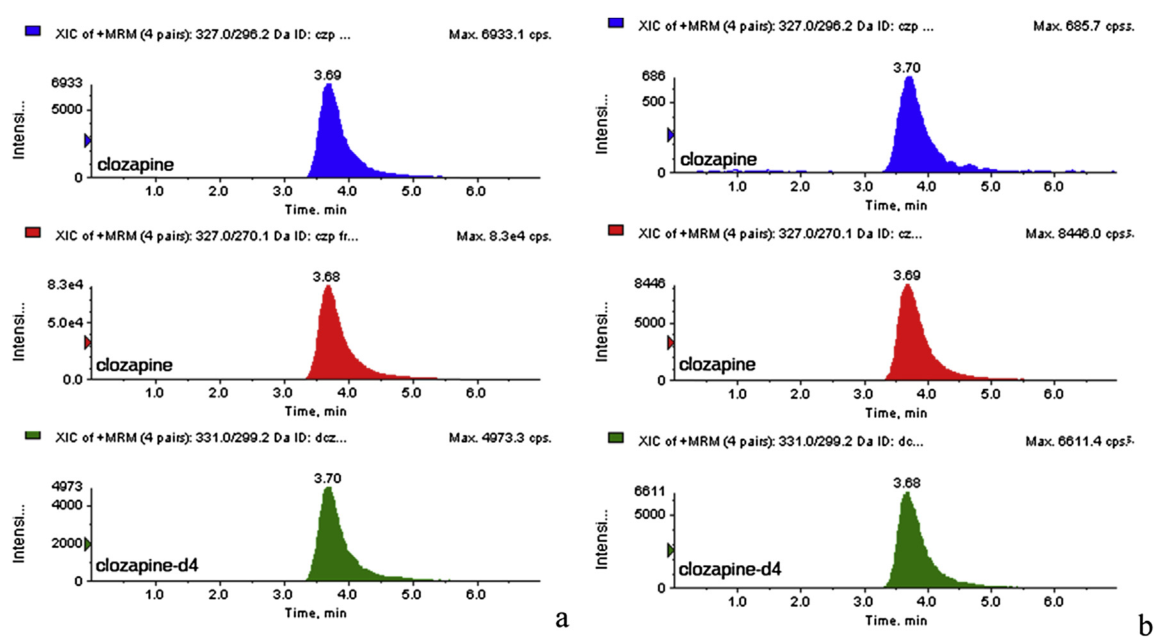

Fig. 2. Selected reaction monitoring chromatograms of 5th segment of hair (4–5 cm) from the deceased (a), and nail from the ring finger of the left foot of the deceased (b).

Table 1
Accuracy and precision for the analysis of clozapine in human hair and nail.

Matrix	Spiked concentration (pg/mg)	Intra-day analysis ($n = 6$)		Inter-day analysis ($n = 6*4$)		Matrix effects (%) ($n = 6$)
		Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	
Hair	50	110.7	5.0	111.8	2.1	82.5
	200	92.5	2.8	92.2	5.8	88.9
	750	96.9	1.8	101.3	1.5	82.2
Nail	50	110.0	5.6	112.3	5.4	84.5
	200	95.9	7.0	97.5	6.9	88.9
	750	95.0	2.7	93.4	2.8	85.2

4.2. Application

The validated UPLC–MS/MS methods were successfully used to measure the clozapine concentration levels in hair and nails from the bloated immersed remains. The analytic result is shown in Table 3, Figs. 3 and 4.

The results for clozapine in all segments of hair and nail were positive. They showed a history of long term use of clozapine. The police investigated mental patients who lived upstream based on this result and finally made a formal identification of the remain. The suspects were caught and confessed. The crime was reconstructed according to the statements of the suspects: the deceased was a chronic schizophrenia patient with a history of clozapine use. He killed his wife half a year ago and was hostile to his wife's family. One day at the end of the year 2012, he was abducted to the suburbs, where the suspects aggressively tied him up and threw him into the river.

Compared to fluid matrices, like urine and blood, keratinized matrices have practical and indispensable values in postmortem toxicology,[23] especially in the condition of advanced putrefaction.[24] The drug secretion is very stable in keratinized matrices,[23] and this was because of their distinctive epicutcile structure and less water content in keratinized matrices.[25] Numerous postmortem applications have been described in the literature where hair analysis was used to document the cases: opiates can be detected from the famous poet John Keats' hair who lived over one century before.[26] And benzoylecgonine can be detected in the scalps and hairs of Chilean and Peruvian mummies dating from 2000 BC to 1500 AD.[27] Moreover, the clozapine in hair can be detected even from the remain which collected from a humid grave (a suitable condition for putrefaction).[10] Similar to our case, P. Kintz et al. reported a population sub-type identification case of a corpse with no organic material left.[24] In that case, hair analysis revealed the simultaneous presence of antipsychotic drug, and these drugs corresponded to the treatment records of a psychiatric patient who disappeared from a neighbor hospital since 14 weeks ago. But until now, there are few reports about nail application in postmortem toxicology.

The concentration of clozapine in the hair and nails of the deceased was obviously reduced from soaking in the water for such a long time. The concentration of clozapine in the hair was 145.4–686.3 pg/mg. This value is significantly lower than in the other studies (Table 4). We suspect that is due to the characteristic that keratinous biological materials could swell when soaked in water for a long time.[28] It can be seen in the W/K ratio in this paper: the hair and nail samples were coated with a thin layer of river sand, and we speculate that some of clozapine was released from the keratinous biological materials and could exist in the sand. From this measurement, the observation that the W/K ratio of 1st segment of hair was higher than the 6th segment can be explained because there was more sand around the hair root than at the hair ends. The W/K ratios of the aqueous wash residue were obviously higher than those in organic wash residue. This discrepancy was found because the sand was scoured off by the water. Consequently, soaking in water for such a long time can cause the loss of clozapine that accumulated in keratinized matrices.

In most cases, the segmental concentrations decreased from proximal to distal[20,29,30]; due to more drug incorporation into proximal portion of the hair through sweat and other secretions,[31] and more reduction of the drug in the distal portion after long-term cosmetic treatments.[32,33] But for this case, clozapine distribution along the hair shaft is unusual. There are two major possible causes for this unusual pattern. First, there are some differences in soaking depth and time for the segments of the hair shaft in water. Second, the drug history is not available in this case. No records shown if the victim still kept his dosage of clozapine before the incident.

The concentration of clozapine in the hair was higher than in the nail. This result was consistent with our previous study.[11] In vitro

Table 2
Calibration curves and range of clozapine in hair and nail.

	Linearity range (pg/mg)	Slope	Intercept	Correlation coefficient	% RSD
Hair	10–1000	0.0074 ± 0.0003	−0.2658 ± 0.0574	0.9986	2.33
Nail	10–1000	0.0064 ± 0.0004	−0.2533 ± 0.0478	0.9987	2.98

Table 3
Concentration of clozapine in different matrix of real sample.

			W/K ratio of aqueous wash residue	W/K ratio of organic wash residue	Concentration in sample (pg/mg)
Hair segment (cm)	0–1		0.40	0.08	160.7
	1–2		0.54	0.17	145.4
	2–3		0.25	0.05	211.1
	3–4		0.15	0.03	288.5
	4–5		0.05	0.01	445.1
	5–6		0.01	0.01	686.3
Fingernail	Left hand (finger)	1[a]	0.10	0.01	125.6
		2	0.06	0.00	184.1
		4	0.09	0.01	89.0
		5	0.05	0.01	106.5
	Right hand (finger)	1	0.03	0.01	132.8
		2	0.10	0.01	126.5
		3	0.12	0.01	120.2
		4	1.09	0.23	538.5
		5	0.01	0.00	119.3
Toe nail	Left foot (toe)	1	0.03	0.00	128.3
		2	0.00[b]	0.00	130.1
		3	0.00	0.01	76.7
		4	0.02	0.00	64.6
	Right foot (toe)	1	0.21	0.02	89.4
		2	0.03	0.01	79.6
		4	0.01	0.00	102.2

[a] 1 to 5 representative of the thumb, index finger, middle finger, ring finger, and little finger in turn.
[b] W/K ratio is less than 0.00.

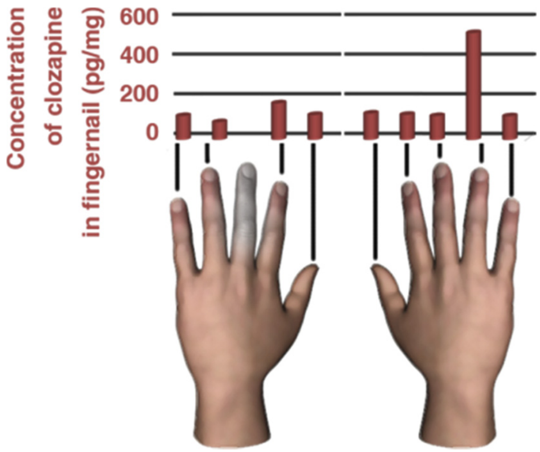

Fig. 3. Diagram of the concentration of clozapine in different finger nails.

binding assays of keratinous materials have a major influence on proteins, lipids, and melanin. For most drugs, the levels in hair were higher than those in nails.[1] Based on our experience, this difference in the levels is due mainly to the melanin in hair. Melanin is a type of anionic polymer; it has a binding affinity for those alkaline molecules. Because most drugs are alkaline, the lipophilicity and membrane permeability of clozapine is considered to be the same in hair and nails. Consequently, melanin was considered an important factor that leads to a higher concentration of clozapine in the hair than in the nails.

The mean concentration in the finger nails was 125.5 pg/mg (except for the ring finger of the right hand), while it was 95.8 pg/mg in the toe nail. RC Irving[1] has analyzed more than eight types of psychotropic drugs between the right and left hands. For the same target drug, there was no significant difference between the left and right samples. In this paper, the concentration of clozapine in the different fingers or toes seemed consistent. Unexpectedly, the concentration of clozapine in the ring finger of the right hand was significantly higher than the concentration in the other fingers. No reference can explain this phenomenon. May be it can be attributed to the personal method of administering the drug, but this hypothesis is simply speculation. In this paper, the concentration in the finger nails was higher than in the toe nails (Student's t test, $P < 0.05$). This observation is consistent with previously reported data,[34] but one paper that found the opposite result.[35] This difference may be caused by the shape and size of the nails, sweat contamination, frequency of washing and blood supply to the tips of the fingers or toes. Additional studies are required to elucidate the mechanism of this phenomenon.

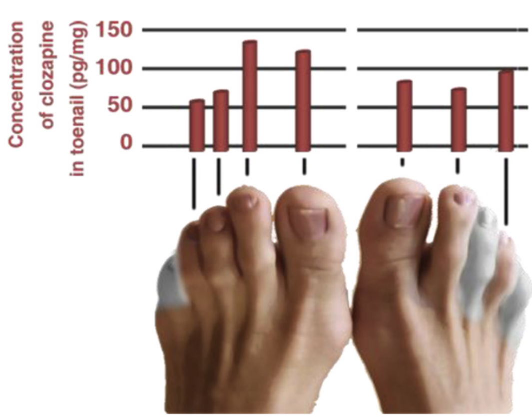

Fig. 4. Diagram of the concentration of clozapine in different toe nails.

Table 4
Concentration of clozapine in hair in different reference.

Number of cases	Concentration in hair (pg/mg)	Dose (mg/day)	Color of the hair	Ethnicity	Reference
16	16 700–59 200	150–425	Blank	Asian	12
1	<1400	Unknown	Black	Asian	9
26	170–34 240	200–700	Unknown	Unknown	7
3	9200/4700/6200	150/300/400	Black/medium brown/light brown	Unknown	8

5. Conclusion

This paper describes an UPLC–MS/MS method for the determination of clozapine in human hair and nails. Clozapine was detected in the hair and nails of a bloated immersed cadaver. The results indicated that the hair and nail samples came from a long term clozapine user. This finding helped police narrow the scope of their investigation and improve the efficiency of solving the case. This paper demonstrates an active and useful role of keratinous biological materials in the identification of an unidentified body with a history of drug use.

Ethical approval

None.

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Conflict of interest

Authors have no financial or personal conflict of interest regarding this manuscript.

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